Clin Exp Immunol 2002; 128:21–26

Leptin inhibits stress-induced apoptosis of T lymphocytes

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(Accepted for publication 20 December 2001)

SUMMARY

Leptin, which is secreted by adipocytes, the placenta and the stomach, not only controls appetite through leptin receptors in the hypothalamus but also regulates cell-mediated immunity. In this study we have demonstrated that continuous injection of leptin prevents the reduction in lymphocyte numbers normally observed in fasted and steroid-injected mice. Consistent with leptin-induced protection, we observed up-regulation of the bcl-xL gene as a result of signal transduction via leptin receptors on lymphocytes. We suggest that leptin might contribute to the recovery of immune suppression in malnourished mice by inhibiting lymphocyte apoptosis.

Keywords apoptosis bcl-xL corticosteroid leptin

INTRODUCTION

Nutritional status and immune function are closely related [1–3]. Food deprivation leads to impaired immune responses and an increase in the incidence of infectious disease, although the mechanism by which this occurs has yet to be elucidated. Adipose tissue preserves energy homeostasis through the storage of triglycerides. However, it has been found recently that a number of cytokine-like molecules, such as leptin [4], tumour necrosis factor-α (TNF-α) [5] and plasminogen activator inhibitor-1 (PAI-1) [6] are secreted from adipocytes, suggesting that adipose tissue may also play a role in the regulation of the immune and haematopoietic systems.

Leptin is secreted specifically by adipocytes [4], and serum leptin levels are proportional to body mass index. However, the placenta [7] and stomach [8] provide additional sources of leptin. Leptin decreases food intake, increases energy expenditure and reduces body weight via leptin receptors within the ventromedial hypothalamus [9], where leptin functions to inhibit the production of neuropeptide Y which stimulates food intake [10]. The murine leptin and leptin-receptor mutants *ob/ob* and *db/db*, respectively, serve as animal models of obesity, and develop marked obesity and diabetes due to deficiencies in leptin signalling [11]. In contrast, leptin transgenic mice with elevated plasma leptin concentrations lack brown or white adipose tissue, show reduced food intake, and are markedly lean in comparison with non-transgenic littermates [12].

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The leptin receptor is expressed in peripheral tissues such as the kidney, lung and adrenal gland [13,14], and several *in vitro* studies have demonstrated that leptin acts directly on the leptin receptor [15,16]. There are at least five splice variants of the leptin receptor Ob-Ra–Ob-Re, and one of these five variants, Ob-Rb, possesses a long intracellular domain demonstrating homology with gp130, a subunit of the IL-6 family of cytokine receptors [17]. On the other hand, Ob-Ra, one of the shortest forms of the leptin receptor, lacks the STAT3 activation domain and is not considered essential for signal transduction [18].

Recent studies have revealed that Ob-Rb is expressed in fetal liver haematopoietic precursor cells, bone marrow and peripheral T cells [14,19]. In adult human bone marrow, both CD34 positive and negative cells express leptin receptor. These findings suggest the possibility that leptin not only regulates body weight, but also modulates the immune system. Indeed, leptin increases the proliferation of haematopoietic stem cell populations at the multilineage progenitor level [18], enhances alloproliferative mixed-lymphocyte reactions, and reverses cellular immune function in fasted mice [20]. In addition, leptin might act as a growth factor for both myeloid leukaemic cells [21] and lung cancer cells [22]. In addition, human white blood cell counts are correlated with body mass index and serum leptin levels [23]. Moreover, diminished cell-mediated immunity and decreased lymphocyte counts have been reported in *ob/ob* and *db/db* mice [24,25].

We demonstrate here that leptin receptor messenger RNA is expressed in lymphoid tissue, and that leptin both restores the decrease in lymphocyte numbers normally observed in fasted mice and prevents apoptosis of lymphocytes in steroid-injected mice. Consistent with the observed anti-apoptotic effect of leptin, we observed up-regulation of the bcl-xL gene by leptin. We suggest that leptin may contribute to recovery of immune

suppression in malnourished mice by inhibiting lymphocyte apoptosis.

MATERIALS AND METHODS

Mice and reagents

Female C57BL/6 mice (6 weeks old) and C57BL/Ks db/db (6 weeks old) were purchased from SLC (Kyoto, Japan) and CLEA (Tokyo, Japan), respectively. Leptin transgenic mice were bred in our laboratory [12]. Recombinant mouse leptin was purchased from R&D Systems (Minneapolis, USA). Hydrocortisone phosphate was purchased from Banyu Pharmaceutical Co. (Tokyo, Japan). All monoclonal antibodies (MoAbs), including hamster anti-CD3 MoAb (2C11), antimouse bcl-2 MoAb and rat antimouse bcl-x MoAb, were purchased from Becton-Dickinson (Franklin Lakes, USA). N-acetylsphingosine (C2-ceramide) was purchased from Sigma Aldrich Japan (Tokyo, Japan).

RT-PCR

Total RNA was extracted from cells using TRIzol® reagent (GIBCO/BRL, Rockville, USA). Five micrograms of total RNA was reverse-transcribed and PCR was performed using specific Ob-Ra and Ob-Rb primers [13].

Fasting and steroid injection experiments

Mice were kept without food for 60 h, with repeated intraperitoneal injection of 0, 1 or 10 μg/g initial mouse body weight of leptin in a solution of phosphate buffered saline (PBS) every 12 h. In a second experiment, 200 μg/g mouse body weight of hydrocortisone phosphate was administered intraperitoneally; 10 μg/g body weight of leptin was injected 2 h before and 4 h after administration of hydrocortisone phosphate. Mice were sacrificed 24 h after hydrocortisone injection.

Cell culture

1×10^5 cells/ml of T cell hybridoma A3·4C6 [26] were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 0·25 μg/ml amphothericin B, 2 mM L-glutamine and 0·05 mM 2-mercaptoethanol, with or without recombinant mouse leptin, under conditions of 37°C and 5% CO_2 for 24 h. In the steroid-induced apoptosis experiment, cells were cultured with 10^{-6} M hydrocortisone for 24 h. In the ceramide-induced apoptosis experiment, cells were cultured with 10 μg/ml C2-ceramide for 18 h. In another experiment, cells were cultured for 5 h on 96-well plates (Costar 3590), coated with anti-CD3 antibody (10 g/ml) overnight.

Assay for DNA fragmentation

Agarose gel electrophoresis was carried out as previously described [27]. DNA was extracted from 1×10^6 cells/sample and suspended in 20 μL of lysis buffer (10 mM EDTA, 50 mM Tris HCl (pH 8·0), 0·5% sodium-N-lauroylsarcosinate) and 1 μL of 10 mg/ml RNaseA was added. Samples were then incubated at 50°C for 30 min, 1 μL of 10 mg/ml Proteinase K was added and incubated for 30 min at 50°C. Then, samples were analysed by agarose gel electrophoresis.

Cell staining

For cell surface analysis, 1×10^5 thymocytes were stained with FITC-conjugated anti-CD4 MoAb and PE-conjugated anti-CD8 MoAb and analysed by FACSCalibur® (Becton Dickinson), as described previously [27]. For detection of bcl-2 and bcl-xL gene products, cells were washed and fixed in PBS containing 4% paraformaldehyde for 20 min. Cells were then washed and suspended in staining buffer (1% BSA in PBS with 0·1% sodium azide) with 0·1% saponin, followed by incubation with hamster antimouse-bcl-2 MoAb or rat antimouse bcl-xL MoAb for 30 min on ice. After washing, cells were stained with FITC-conjugated antihamster IgG or FITC-conjugated antirat IgG, respectively, for 30 min on ice. After washing, cells were analysed by FACSCalibur®. In order to detect apoptosis, cells were stained with 50 μg/ml of propidium iodide after fixation with 70% ethanol for 4 h at 4°C and treatment with 100 μg/ml of RNaseA. After washing, apoptotic cells were determined as the proportion of hypodiploidal cells.

Northern blotting

Total RNA was extracted from A3·4C6, spleen and thymus cells using TRIzol® reagent. Samples of 15 μg of RNA were applied to each lane. After electrophoresis, RNA was transferred to a nylon membrane and hybridized with a ^{32}P-labelled mouse bcl-xL specific cDNA probe. Autoradiography was performed for 24 h and analysed using BAS 2000® (Fuji Photo Film, Tokyo, Japan).

RESULTS

To clarify the involvement of leptin in the immune response, we first confirmed the expression of leptin receptor messenger RNA in lymphoid tissue. Both short and long isoforms of the leptin receptor, Ob-Ra and Ob-Rb, were detected in the spleen, thymus and bone marrow by RT-PCR (Fig. 1).

Next, we investigated the effect of leptin on the number of lymphocytes detected in fasted mice. After 60 h of fasting, decreased numbers of thymocytes and splenocytes were detected (Table 1). In the thymus, the $CD4^+CD8^+$ T cell population decreased dramatically from 85% (control) to 25% in fasted mice (Fig. 2a). On the other hand, the proportion of CD4 and CD8 single-positive T cells did not differ among the spleens of fasted and fed control mice (data not shown). Repeated injection of leptin reduced the observed decrease in lymphocyte numbers within the thymus of fasted mice, particularly that of the $CD4^+CD8^+$ T cell population (Table 1, Fig. 2a). The protective effect of leptin was observed to be dose-dependent (Table 1, Fig. 2a). However, one area that remained unclear was whether leptin exerted a direct effect on lymphocytes, or whether it exerted its effect through an indirect mechanism, for example, by counteracting the endocrinological disturbances associated with fasting.

Fasting is widely known to cause the stress-induced release of several hormones *in vivo* [28]. In particular, a steroid hormone derived from the adrenal glands is suspected to play an important role in fasting-induced lymphopenia because lymphocyte numbers do not decrease in adrenectomized mice, even during fasting [29]. To investigate the effect of leptin on the steroid-induced cell death of lymphocytes, hydrocortisone was intraperitoneally injected (200 μg/g body weight) into mice, with or without leptin (10 μg/g body weight). As noted during fasting, steroid injection decreased the number of lymphocytes, particularly $CD4^+CD8^+$T lymphocytes, within the thymus. Injection of leptin inhibited the steroid-induced decrease of thymocytes and splenocyte numbers (Table 1) and reversed the observed decline

in CD4⁺CD8⁺ thymocyte numbers (Fig. 2b). This result is consistent with the observed effect of leptin in fasted mice. Moreover, DNA fragmentation of thymocytes in steroid-injected mice was prevented by leptin administration (Fig. 3). In contrast, when leptin-receptor-defective *db/db* mice were treated with hydrocortisone, the proportion of CD4⁺CD8⁺ T cells within the thymus decreased, as was observed for normal mice, but the change was not reversed by injection of leptin (Fig. 2c). This result indicates that the protective effect of leptin against the decline in lymphocyte numbers is related specifically to the binding of leptin to its receptor. When combined, these results strongly suggest that leptin can reverse the decline in T cell numbers due to fasting by preventing steroid-induced apoptosis of lymphocytes by binding to its receptor.

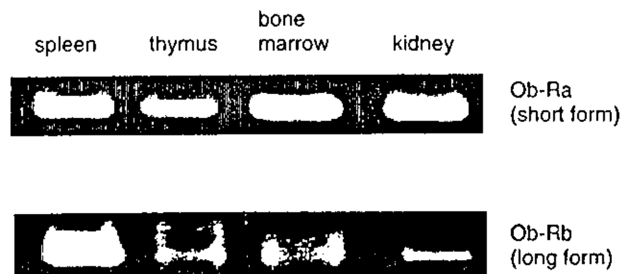

Fig. 1. The leptin receptor is expressed in lymphocytes. Short and long isoforms of the leptin receptor, Ob-Ra and Ob-Rb, were detected by RT-PCR in lymphocytes of the spleen, thymus and bone marrow. Kidney served as a positive control for expression of the leptin receptor.

Table 1. Leptin recovers the decrease in lymphocytes observed in starved and steroid injected mice

	Thymus		Spleen
Treatment	Thymocytes	Double positive thymocytes	Splenocytes (×10⁶)
Untreated	177.0 ± 20.8	154.0 ± 25.4	125.0 ± 4.80
Starved	18.2 ± 11.5	5.12 ± 5.29	15.7 ± 6.15
Starved and leptin treated (1 µg/g)	25.8 ± 10.1	10.1 ± 6.20	28.1 ± 10.3
Starved and leptin treated (10 µg/g)	45.2 ± 3.79	27.7 ± 4.22	50.8 ± 8.29
Untreated	101.0 ± 6.42	83.0 ± 5.26	159.0 ± 66.1
Hydrocortisone treated	12.1 ± 5.25	2.39 ± 3.00	65.0 ± 16.3
Hydrocortisone and leptin (10 mg/g) treated	24.1 ± 5.30	9.87 ± 5.21	102.0 ± 13.1

The mice were treated as described in the Materials and methods section. Data are represented as the mean ± s.d., $n = 3$ per group.

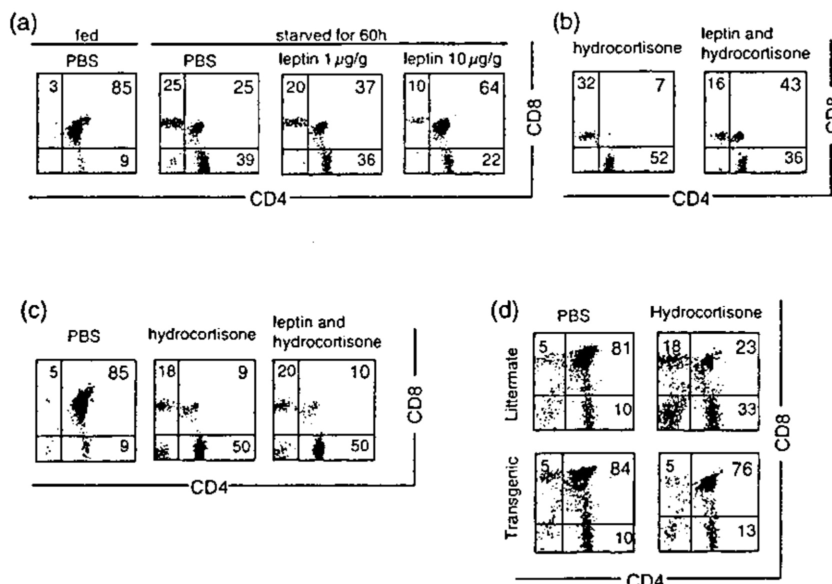

Fig. 2. (a) Leptin reverses the decrease in CD4⁺CD8⁺ thymocytes normally observed in fasted mice. (b) Leptin protects against steroid-induced apoptosis of CD4⁺CD8⁺ thymocytes. (c) Leptin cannot rescue thymocytes of *db/db* mice from steroid-induced apoptosis. (d) Thymocytes of leptin transgenic mice are resistant to steroid-induced apoptosis. This figure represents one of three separate experiments in which similar results were obtained. The percentage of fluorescence-positive cells that were detected is indicated in the corresponding squares.

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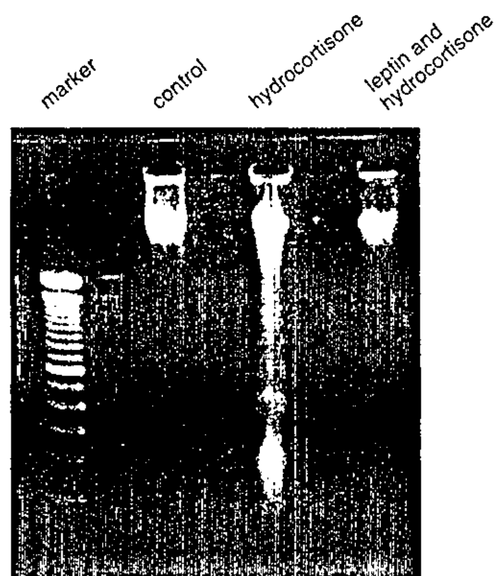

Fig. 3. Leptin prevents DNA fragmentation in thymic T cells induced by steroid administration.

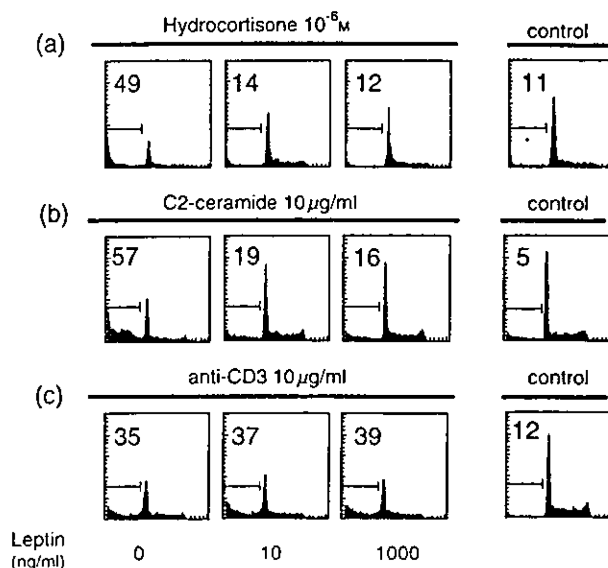

Fig. 4. Leptin prevents apoptosis induced by steroid (a) and ceramide (b), but does not prevent that induced by anti-CD3 MoAb (c). The T cell hybridoma, A3·4C6, was preincubated with or without leptin and apoptosis was induced. The proportion of apoptotic cells was analysed by FACSCalibur® and is shown in figures.

Next, we used leptin transgenic mice to confirm the anti-apoptotic effect of leptin on thymocytes. Plasma leptin concentrations in leptin transgenic mice are approximately 12-fold higher than those of non-transgenic littermates [12]. In the present study, no differences in the surface markers of lymphocytes in the spleen, thymus and bone marrow were observed between leptin transgenic mice and non-transgenic littermates (data not shown). However, when hydrocortisone was injected into leptin transgenic mice and non-transgenic littermates, lower levels of $CD4^+CD8^+$ thymocytes were not found in the leptin transgenic mice compared to non-transgenic littermates (Fig. 2d), suggesting that the thymocytes of leptin transgenic mice are protected from steroid-induced apoptosis by maintaining elevated plasma leptin levels.

To clarify the mechanism by which leptin prevents steroid-induced apoptosis, we performed *in vitro* experiments using a murine T cell hybridoma, A3·4C6 [26]. This hybridoma has been shown to be specific for sperm whale myoglobin and to express the leptin receptor (data not shown). After incubation with leptin for 24 h, followed by an additional 24 h with hydrocortisone, apoptotic cells were examined by flow cytometric analysis. Leptin decreased the proportion of apoptotic cells in a dose-dependent manner (Fig. 4a). In contrast, the anti-CD3 antibody-induced apoptotic death of T cells was not prevented by leptin (Fig. 4c), suggesting that leptin is involved in steroid-induced apoptosis but not in activation-induced cell death.

Recent studies have revealed that ceramide is likely to be one of the several second messengers involved in steroid-induced apoptosis [30]. To investigate whether leptin is effective in preventing ceramide-induced cell death, apoptosis of A3·4C6 induced by N-acetylsphingosine (C2-ceramide) was examined. Leptin decreased the number of apoptotic cells induced by ceramide in a dose-dependent manner (Fig. 4b), as it did following steroid-induced apoptosis (Fig. 4a). These results suggest the possibility that leptin prevents steroid-induced apoptosis downstream of ceramide.

As Ob-Rb displays homology with gp130, a common signal transduction molecule, leptin is thought to activate STAT3 in the same way as does gp130 receptor signalling [16]. Recent studies have revealed that STAT3 binds to the promoter region of bcl-xL, an antiapoptotic molecule of the bcl-2 family, and thereby enhances transcription of the protein [31]. We therefore investigated the possibility that the anti-apoptotic activity of leptin can be attributed to increased expression of bcl-xL following activation of STAT3. We performed flow cytometric analysis and Northern blotting to confirm bcl-xL expression at both the protein and messenger RNA level. When the T cell hybridoma A3·4C6, expressing Ob-Rb, was incubated in the presence of leptin for 24 h, expression of cytoplasmic bcl-xL protein was significantly increased in a dose-dependent manner (Fig. 5a). Moreover, bcl-xL messenger RNA in A3·4C6 was enhanced by leptin in a dose-dependent manner (Fig. 5b). Consistent with the results of *in vivo* experiments, bcl-xL messenger RNA was enhanced within the spleen and thymus of leptin-injected mice (Fig. 5c).

DISCUSSION

The mechanism by which malnutrition leads to immunodeficiency is not fully understood. However, leptin has been reported to be the key link between fasting and immunodeficiency [20], and to inhibit thymocyte apoptosis both *in vivo* and *in vitro* [32]. As fasting increases the production of steroid hormones, it may induce apoptosis of T cells. The present study offers compelling evidence that leptin inhibits the decline in lymphocyte numbers that normally accompanies fasting, in addition to T cell apoptosis following steroid injection. The evidence further suggests that leptin protects lymphocytes through up-regulation of bcl-xL via leptin receptors on lymphocytes. Only one-third of T cell hybridoma cells expressed bcl-xL after leptin stimulation and the observed increase in expression of the bcl-xL gene was not as dra-

matic as that observed following leptin stimulation of fasted mice (Fig. 5). These data suggest that bcl-xL alone is not responsible for the observed inhibitory effect of leptin. Fasting increases the level of steroid hormone within the serum and decreases the amount of circulating leptin [28], both of which can accelerate apoptosis of lymphocytes and impair the immune response.

In contrast to a previous report that found an association between obesity, immune suppression and the presence of infectious disease [33] and cancer [34], our results suggest that obesity might confer resistance to infection. This paradoxical observation can be explained by considering the possibility that the leptin signal is less transducible in obese patients.

Ceramide is generated by hydrolysis of sphingomyelin followed by activation of acidic sphingomyelinase. It functions as an intracellular second messenger, mediating the sphingomyelin signalling pathway. Recently, ceramide has been examined as a common intermediator of several apoptotic stimuli, including steroid administration [30]. However, it is not involved in Fas-induced apoptosis [35,36]. The apoptotic effect of ceramide is due to its induction of cytochrome c release from mitochondria [37]. The bcl-2 gene family can inhibit ceramide-induced apoptosis [38,39].

The present study confirms the findings of reduced numbers of lymphocytes in fasting, and illustrates that leptin prevents the decline in lymphocytes during fasting, in addition to preventing apoptosis of T cells following steroid injection [32]. This may be explained partly by the ability of leptin to up-regulate bcl-xL through the leptin receptors on lymphocytes. However, leptin might not be able to rescue a T cell hybridoma from activation-induced cell death through CD3 stimulation, which is known to involve Fas and the Fas ligand [40–42]. The different effects of leptin on steroid-induced apoptosis and activation-induced cell death might be explained by the bcl-2 related gene products, which prevent apoptosis downstream of ceramide.

Leptin was found originally to modulate body weight but, more recently, it has become recognized as an immune regulator. Administration of leptin to fasted mice has been observed to reverse impairment of T cell function [20]. Although the present study illustrates that leptin inhibits the stress-induced apoptosis of T cells, other effects with regard to immunity remain unclear.

As shown in the present study, lymphocytes can be preserved during fasting by administration of exogenous leptin. This suggests that leptin might be used therapeutically to treat immunodeficiency caused by severe malnutrition and cachexia in cancer and AIDS patients. Such applications may be assessed by further investigation using mouse models of AIDS [43]. On the other hand, it is possible that the anti-apoptotic effects of leptin might result in autoreactive T cells and lead to the development of autoimmune disease. The possibility that leptin might lead to autoimmunity is currently under investigation using our leptin transgenic mice.

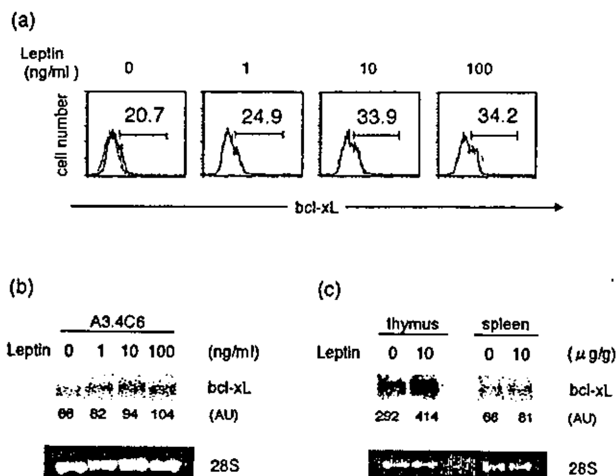

Fig. 5. (a) Leptin induced bcl-xL protein in the T cell hybridoma. The T cell hybridoma, A3·4C6 was incubated in the presence or absence of leptin for 24 h. Expression of the bcl-xL gene was detected with cell-staining using antibcl-xL MoAb and a fluorescent conjugated second antibody. The dotted line indicates cell staining using the fluorescent conjugated antibody without antibcl-xL MoAb. (b) Expression of bcl-xL messenger RNA is enhanced by leptin. A3·4C6 were incubated with graded concentrations of leptin for 24 h and RNA was extracted as described in the materials and methods section. (c) C57/BL6 mice were injected with either PBS or leptin (10 μg/g) and RNA was extracted from the thymus and spleen 24 h later. Northern blotting was performed as described in the materials and methods section. The 28S ribosomal RNA bands were stained with ethidium bromide and are shown in the lower panels of this figure. The relative band intensities of bcl-xL are indicated by assignment of arbitrary units (AU).

ACKNOWLEDGEMENTS

We thank K. Ohmura for his excellent technical assistance. This work was supported by grants from the Ministry of Education, Science and Culture of Japan.

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Reduction of Serum Soluble CD26/Dipeptidyl Peptidase IV Enzyme Activity and Its Correlation with Disease Activity in Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** CD26 is the cell surface activation antigen with dipeptidyl peptidase IV (DPPIV) enzyme activity at the extracellular domain that is preferentially expressed on memory T cells and has a role in T cell immune responses. The soluble form of CD26 is present in serum and recombinant soluble CD26 (rsCD26) can enhance *in vitro* antigen-specific T cell responses. To determine the role of soluble CD26 (sCD26) in the pathophysiology of patients with systemic lupus erythematosus (SLE), we measured levels of sCD26 and its specific DPPIV activity in serum.

Methods. Serum sCD26 levels and DPPIV activity were measured by sandwich ELISA in 53 patients with SLE and 54 healthy controls. Serum sCD26 was identified by immunoprecipitation and immunoblot analysis. Expression of CD26 on T cells was analyzed by flow cytometry.

Results. Serum levels of sCD26 and its specific DPPIV activity were significantly decreased in SLE and were inversely correlated with SLE disease activity index score, but not with clinical variables or clinical subsets of SLE. Close correlation between sCD26/DPPIV and disease activity was observed in the longitudinal study.

Conclusion. Serum levels of sCD26 may be involved in the pathophysiology of SLE, and appear to be useful as a new disease activity measure for SLE. (J Rheumatol 2002;29:1858–66)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS
DIPEPTIDYL PEPTIDASE IV
SOLUBLE CD26
DISEASE ACTIVITY

T cell activation antigen CD26 is a 110 kDa cell surface glycoprotein with diverse functions[1-4]. Although constitutively expressed in the kidney proximal tubules, intestine, and bile duct, the expression level of CD26 on T cells is tightly regulated and its density is markedly enhanced after T cell activation[5,6]. In the resting state, CD26 is expressed on a subset of CD4 memory T cells and this CD4+ CD26^{bright} T cell population has been shown to respond maximally to recall antigens and has high migratory capacity[2,7,8].

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Supported by US National Institutes of Health grant AR33713; and by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology, and from the Ministry of Health, Labor and Welfare, Japan.

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Submitted August 21, 2001; revision accepted March 7, 2002.

CD26 contains dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5) enzyme activity in its extracellular domain that can cleave aminoterminal dipeptides with either proline or alanine in the penultimate position[4,5]. Recently, our group and others have shown that CD26/DPPIV can cleave RANTES (regulated on activation, normal T cell expressed and secreted)[9-11] and stromal cell derived factor-1 (SDF-1)[12,13] and modify their original functions. CD26 is not only highly expressed on activated T cells but also is involved in the process of T cell costimulation[14,15]. There is evidence that CD26 interacts with both CD45, a protein tyrosine phosphatase, and adenosine deaminase, each capable of functioning in a signal transduction pathway[16-18]. We recently demonstrated that CD26 binds the mannose 6-phosphate (M6P)/insulin-like growth factor (IGF) II receptor via M6P residues in the carbohydrate moiety of CD26, the interaction that plays an important role in CD26 mediated T cell costimulation[19]. We showed that recombinant soluble CD26 (rsCD26) could enhance T cell proliferative response against memory antigens, with DPPIV enzyme activity being necessary for its enhancing effect *in vitro*[20,21]. Moreover, correlation between serum sCD26/DPPIV level and the relative responsiveness of the memory antigen driven T cell activation to exogenous sCD26 suggests that serum sCD26 modulates T cell immune response *in vivo*.

Systemic lupus erythematosus (SLE) is a multisystem

inflammatory disease caused by tissue damage resulting from autoantibody and complement fixed immune deposition. Abnormal immune activation, such as hyperactivity of T and B cells, may cause infiltration of lymphocytes into the tissue, leading to increased serum cytokine levels and production of a variety of autoantibodies. In this regard, aberrant expression of immune costimulatory molecules may contribute to the pathophysiology of SLE. Studies indicate that blood lymphocytes of patients with SLE often express higher levels of immune accessory molecules such as CD80[22,23], CD86[24], CD95[25,26], and CD154[27,28] than those of healthy individuals. Further, it has been reported that levels of soluble forms of T cell activation antigens as well as cell adhesion molecules, including sCD25[29,30], sCD23[31], sCD27[30], sCD95[32], sVCAM-1[33-36], sICAM-1[37], and sE-selectin[38], were all increased in the serum of patients with SLE, having strong correlation with SLE disease activity. More recently, plasma levels of soluble CD40 ligand (CD154) were significantly higher in patients with SLE than in controls, and they were associated with SLE disease activity[39].

On the other hand, it was reported that serum DPPIV enzyme activity was consistently decreased compared to healthy controls regardless of SLE disease activity[40-42], although these studies did not detect serum CD26 levels. Since there are several enzymes that have DPPIV-like enzyme activity, such as aminopeptidase P[43], DPPT-L[44,45], FAP-α[46], DPPIV-ß[47,48], and dipeptidyl peptidase II[41], these studies may not accurately quantify specific CD26 associated DPPIV enzyme activity in plasma.

We investigated the levels of sCD26 and its specific DPPIV enzyme activity in the serum of patients with SLE and their relationship with disease status and activity.

MATERIALS AND METHODS

Patients. In this study, 53 patients with SLE (47 women, 6 men, ages 18–79 yrs) and 54 healthy controls were enrolled. Patients were referred to the Research Hospital, Institute of Medical Science, University of Tokyo, or Keio University Hospital, in Tokyo. All patients fulfilled the 1982 revised criteria for the classification of SLE[49]. Active disease was indicated by SLE Disease Activity Index (SLEDAI) score of > 10 points.

Detection of serum sCD26 and DPPIV activity. Serum samples were kept frozen at –80°C until assayed. We confirmed equality of sCD26 levels and DPPIV enzyme activity before and after samples were frozen. We measured serum levels of sCD26 using ELISA, as well as its associated serum DPPIV enzyme activity, as developed in our laboratory[50]. Briefly, Maxisorp ELISA immunoplates (Nunc A/S, Roskilde, Denmark) were coated with 100 µl of 10 µg/ml anti-CD26 Mab (5F8) in 0.05 M carbonate/bicarbonate buffer, pH 9.6, by incubating overnight at 4°C. Remaining binding sites were blocked with 200 µl of 25% Block Ace® (DaiNihon Pharmaceutical, Osaka, Japan) at 4°C overnight. Plates were then incubated with 100 µl of serum appropriately diluted with 0.01 M phosphate buffered saline (PBS) containing 0.05% Tween 20 for 2 h at room temperature. Bound sCD26 molecules were detected by incubating with 10 µl of biotin conjugated anti-CD26 Mab (1F7) followed by ExtrAvidin®-alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA). The plates were developed with 1 mg/ml p-nitrophenyl phosphate in 10 mM diethanolamine buffer, pH 9.6, containing 0.5 mM $MgCl_2$. Color development was monitored at 405 nm on an ELISA reader (model 3550; Bio-Rad Laboratories, Hercules, CA, USA). This immunoassay was standardized by the standard curve using recombinant sCD26 produced by transfected Chinese hamster ovary cells as described[20]. In parallel with sCD26 protein concentration, DPPIV enzyme activity of bound sCD26 to 5F8 was detected by incubation with glycylproline p-nitroanilide (Gly-Pro-p-NA, 1 mg/ml in PBS; Sigma). Color development was measured at 405 nm. These results were standardized by a standard curve for p-nitroaniline (Sigma).

Immunoprecipitation and immunoblotting of soluble CD26. After preclearing human sera from patients with SLE or healthy controls with protein G-sepharose CL-4B (Pharmacia Biotech), aliquots were immunoprecipitated with anti-CD26 Mab 1F7 conjugated protein G-sepharose CL-4B for 3 h at 4°C. After washing 5 times with immunoprecipitation buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40), immunoprecipitates were denatured with sample buffer including 5% 2-mercaptoethanol at 95°C for 5 min. The samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing condition and electroblotted onto PVDF membranes (Millipore, Bedford, MA, USA). The blots were treated with 5% skim milk blocking buffer for 1 h at room temperature. The blots were washed 3 times with TBS-Tween buffer and incubated 1 h at room temperature with biotin conjugated anti-human CD26 Mab (1F7). After 3 washings, the blots were incubated with horse-radish peroxidase (HRP) conjugated streptavidin (Funakoshi, Tokyo, Japan) followed by ECL reagent (Amersham). The blots were then exposed to radiographic film for a few minutes.

Flow cytometry. Mononuclear cells isolated with Ficol-Hypaque were washed twice and suspended in staining buffer containing PBS, 2% human AB serum, 0.05% NaN_3, and 5% Block Ace. Cells were stained for flow cytometry in staining buffer containing saturating amounts of fluorochrome or phycoerythrin conjugated Mab. We used fluorochrome conjugated antibody specific for CD26 (Ta1) and phycoerythrin conjugated Mab specific for human CD3 (UCTH-1) (Beckman Coulter, Fullerton, CA, USA). After 30 min at 4°C, the cells were washed with staining buffer twice, fixed with 1% formaldehyde solution, and analyzed with flow cytometry using a FACScalibur (Becton Dickinson, Immunocytometry Systems, San Jose, CA, USA).

Statistical analysis. The clinical chart of each patient was retrospectively examined for duration and extent of disease, as well as for disease related treatment that was administered up to the time of the assay. Statistical analysis was performed with Student's t test or Pearson's test.

RESULTS

sCD26 levels and DPPIV enzyme activity in the serum of patients with SLE. To determine the clinical significance of serum sCD26 levels in patients with SLE, we measured serum levels of sCD26 as well as its associated DPPIV enzyme activity in 53 patients with SLE and 54 healthy controls. Patients' clinical and demographic characteristics are summarized in Table 1. In this population, the ratio of the clinical subset and positive ratio of antinuclear antibody or anti-DNA antibody were compatible to the general population of patients with SLE. As shown in Figure 1A, serum levels of sCD26 in patients with SLE were significantly lower than in controls (10.5 ± 3.4 vs 15.6 ± 2.4 µg/ml; $p < 0.0001$). Soluble CD26-specific DPPV enzyme activity also showed a significant decrease in SLE patients compared to controls (5.8 ± 1.8 vs 9.3 ± 1.3 µmol/min/l; $p < 0.0001$) (Figure 1B). Moreover, serum levels of sCD26 were significantly correlated with its specific DPPIV enzyme activity (r

Table 1. Clinical and demographic characteristics of patients with SLE (n = 53).

Female %	89
Age at SLE onset, mean ± SD yrs	30.4 ± 14.9
Disease duration, mean ± SD yrs	11.2 ± 8.7
Organ involvement, %	
Kidneys	40
Skin	45
Joints	69
Hematolgic system	72
Lung	29
Heart	13
Central nervous system	1.7
Antinuclear antibodies, %	95
Antibodies to	
Double stranded DNA, %	66
Sm, %	61
RNP, %	55
Low complement, %	56

= 0.945, p < 0.0001, n = 107) (Figure 1C). These results indicate that decreased serum levels of sCD26 and associated DPPIV activity were observed in patients.

Characterization of sCD26 in serum of patients and controls. Our observation of decreased serum levels of sCD26 and DPPIV enzyme activity in patients with SLE suggested the possibility that the sCD26 molecules were modified by the disease itself. To characterize the sCD26 molecules found in the serum of patients and compare them to those seen in healthy controls, we carried out immunoprecipitation and immunoblotting studies by anti-CD26 Mab. Western blotting for sCD26 revealed that an anti-CD26 Mab (1F7) reacted with sCD26 molecules in SLE patients' sera as well as the recombinant sCD26, which has a 28 amino acid deletion at the N-terminal end (intracellular domain of CD26) (Figure 2). The calculated molecular mass of the band in the sera of SLE patients as well as controls was 110 kDa. In addition, results obtained with another anti-CD26 Mab (5F8) used in the ELISA for detection of sCD26 were similar (data not shown). Densitometric analysis showed that the intensity of the bands correlated with serum sCD26 levels (data not shown). These data therefore indicate that the decrease in serum levels of sCD26 in patients with SLE is not due to major protein modification (e.g., glycosylation, immune complex formation or degradation). Rather, it is most likely due to an actual reduction in the amount of sCD26 in the serum.

Relationships between serum levels of sCD26 and associated DPPIV enzyme activity and clinical variables. We next analyzed the relationship between serum levels of sCD26/DPPIV enzyme activity and clinical variables. As illustrated in Table 2, serum levels of both sCD26 and DPPIV enzyme activity showed significant correlation only with the titer of anti-RNP antibody (p < 0.05). In addition, neither serum sCD26 levels nor DPPIV enzyme activity displayed significant correlation with any disease related complications, e.g., butterfly and/or discoid rash, renal disease, arthritis and serositis, neurologic disease, and hematological disease (data not shown). In contrast, as shown in Figure 3, serum sCD26 levels showed a significant correlation with SLEDAI score (r = –0.40, p < 0.005, n =

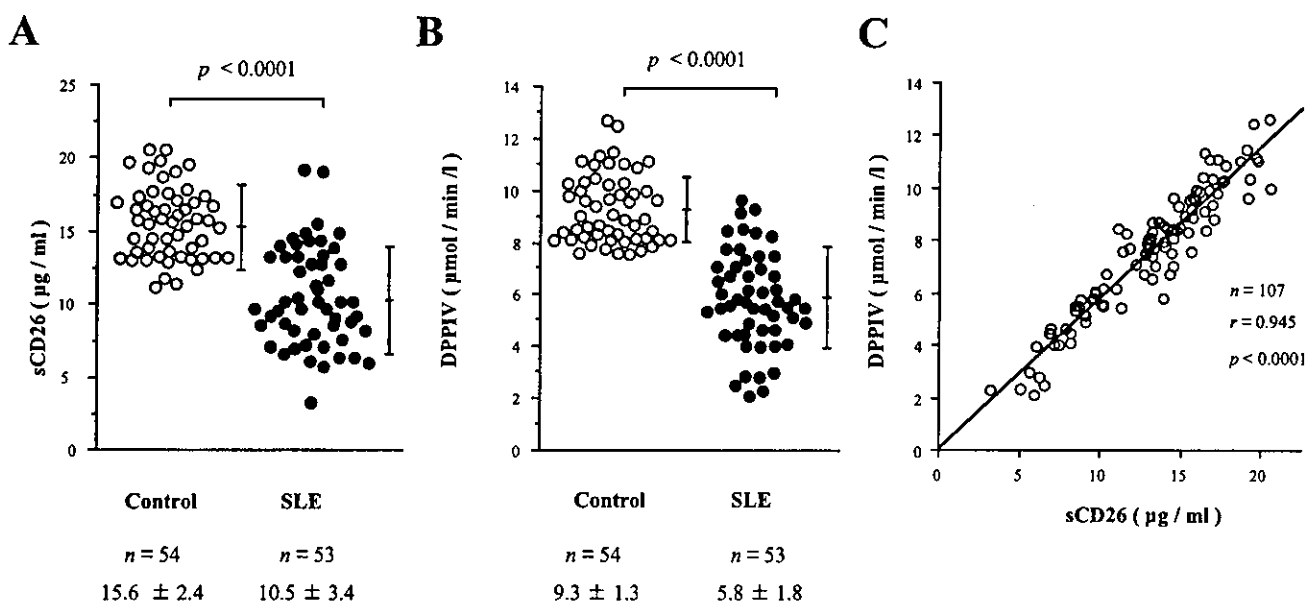

Figure 1. Comparison of serum sCD26 levels and specific DPPIV enzyme activity in controls and patients with SLE. A. Serum sCD26 level and (B) specific DPPIV enzyme activity were measured by sandwich ELISA. Sample number and mean value ± SD shown at the bottom of the figures. C. Correlation between serum sCD26 level and its DPPIV enzyme activity.

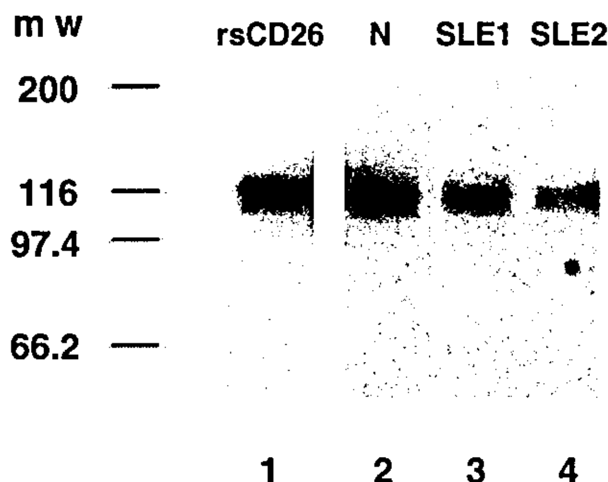

Figure 2. Characterization of sCD26 as a 110 kDa molecule in the serum of healthy controls and SLE patients by immunoprecipitation and immunoblotting analysis. Serum sCD26 was immunoprecipitated with anti-CD26 Mab (1F7) and subject to 6% acrylamide gel SDS-PAGE; the blot was immunoblotted with anti-CD26 Mab (1F7). 100 ng of recombinant soluble CD26 (lane 1), 10 µl of healthy control serum (lane 2) or SLE serum (lanes 3, 4) were applied in each lane. Molecular weight marker shown at left.

Table.2 Correlation between sCD26 or DPPIV and clinical variables ($p < 0.05$ considered significant).

		sCD26 Level		DPPIV Activity	
	n	r	p	r	p
Age at last evaluation	53	0.18	0.22	0.23	0.11
Disease duration at last evaluation	53	0.02	0.90	0.04	0.79
Daily dosage of prednisolone	53	−0.16	0.33	−0.19	0.24
ESR	53	−0.27	0.06	−0.22	0.13
Lymphocyte count	53	0.07	0.66	0.23	0.13
IgG	47	0.15	0.31	0.21	0.16
IgA	47	−0.10	0.50	−0.04	0.82
IgM	47	−0.18	0.23	−0.26	0.08
C3	48	0.04	0.78	0.13	0.37
C4	48	0.00	0.99	0.07	0.66
CH50	44	0.07	0.64	0.14	0.36
Titer of anti-ds-DNA Ab	47	−0.26	0.07	−0.25	0.09
Titer of anti-RNP Ab	31	0.44	0.02	0.44	0.02
Titer of rheumatoid factor	33	0.26	0.16	0.27	0.15

Ab: antibody.

53). In addition, the mean value of serum sCD26 levels in patients with active disease (those with SLEDAI > 10) was significantly lower than in patients with inactive SLE (7.9 ± 2.4 vs 11.3 ± 3.3 µg/ml; $p < 0.005$). Moreover, we serially analyzed serum sCD26 levels. Figure 3C shows the clinical course of a representative case. In this case, serum sCD26 level was already low when disease was inactive in April 1997. As the patient's clinical condition was exacerbated in July 1998, serum levels of sCD26 and its associated DPPIV activity became lower. Of interest is that these levels again recovered when the patient's disease activity subsided, in November 1998. In addition, in July 1998, this patient's clinical condition was complicated with acute hemophagocytic syndrome, which was pathologically confirmed by the presence of mature looking histiocytes scattered among the hematopoietic cells in the specimen obtained from a bone marrow biopsy (data not shown).

Relationships between T cell CD26 expression and serum sCD26 levels. To define the mechanism for the decreased levels of serum sCD26 in patients with SLE, we evaluated the surface expression of CD26 on peripheral blood T cells by flow cytometry. As shown in Figure 4, levels of CD26 positive T cells were significantly lower in SLE patients than in healthy controls (517.6 ± 93.1 vs 1089.5 ± 109.0 cells/mm^3; $p < 0.001$). Moreover, there was significant correlation between CD26 surface expression levels and serum sCD26 levels ($r = 0.56$, $p < 0.005$, $n = 24$). However, it should be noted that the mean fluorescent intensity (MFI)

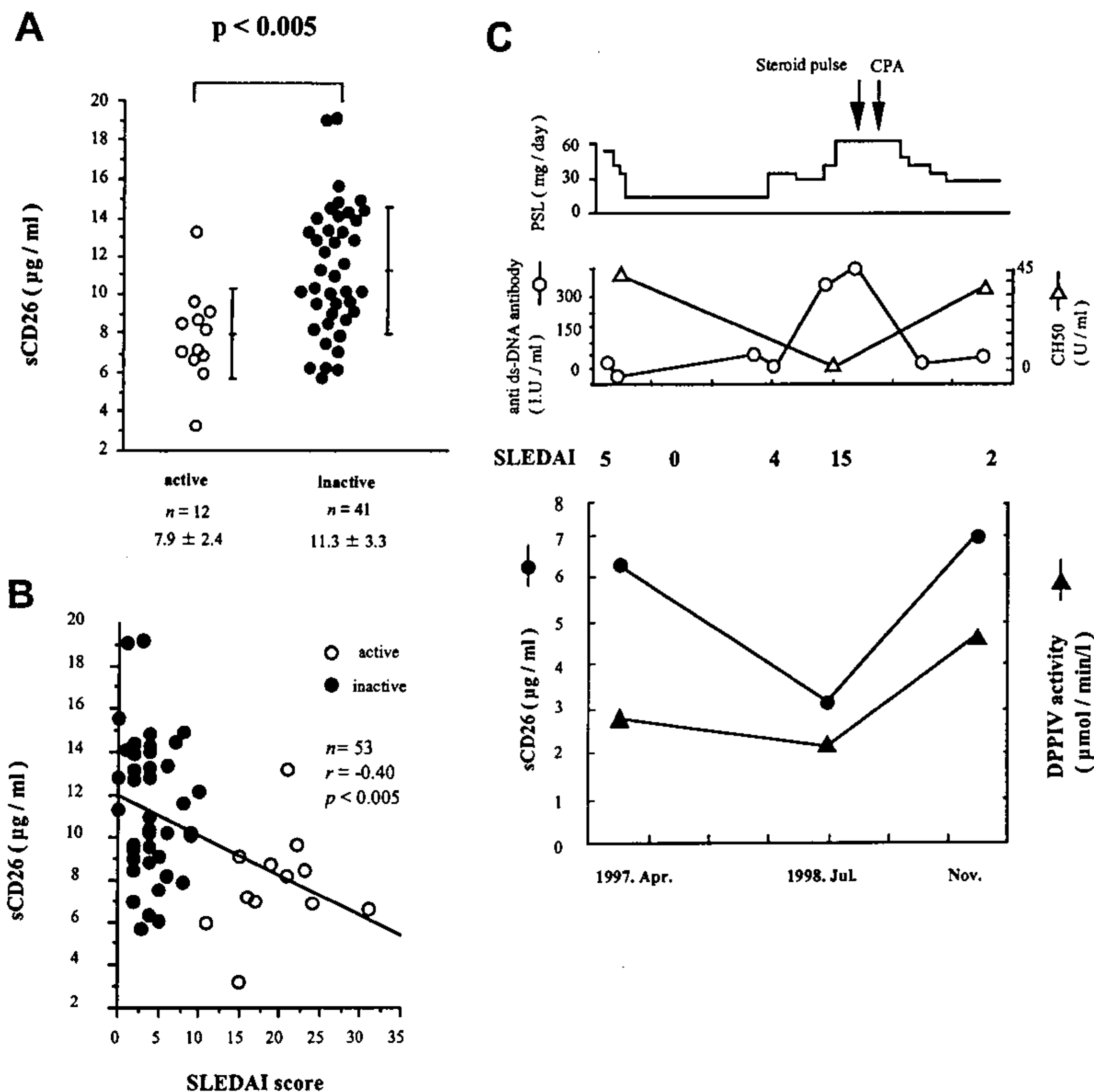

Figure 3. Serum sCD26 levels and DPPIV enzyme activity in SLE patients with active disease. A. Mean value of serum sCD26 levels (patients with SLEDAI > 10) was significantly lower than in patients with inactive disease ($p < 0.005$). Sample number and mean values ± SD shown at the bottom of the figures. B. Serum sCD26 levels showed a significant correlation with SLEDAI score ($r = -0.40$, $p < 0.005$). C. Longitudinal study of a representative case with active disease.

of CD26 expression on T cells from the SLE patients tended to be higher than that of controls (Figure 4B).

DISCUSSION

We determined serum levels of the soluble form of the T cell activation antigen CD26 (sCD26) as well as its associated DPPIV enzyme activity in patients with SLE. We also defined the relationships between sCD26/DPPIV and the various clinical variables of SLE. We found that serum levels of sCD26 and DPPIV enzyme activity were significantly decreased in patients with SLE and were inversely correlated with SLE disease activity.

Reports show that total serum DPPIV enzyme activity was significantly decreased regardless of SLE disease activity[40,42]. However, by focusing only on DPPIV enzyme activity, these studies did not evaluate levels of serum sCD26. More importantly, dipeptide derived chromogenic or fluorogenic substrates were used in assays to define DPPIV enzyme activity. Since there are several enzymes that have DPPIV-like enzyme activity, such as aminopeptidase P[43], DPPT-L[44,45], FAP-α[46], DPPIV-β[47,48], and DPPII[41], these reports may not accurately quantify specific CD26 associated DPPIV enzyme activity in serum. The comparison between specific CD26 associated DPPIV enzyme

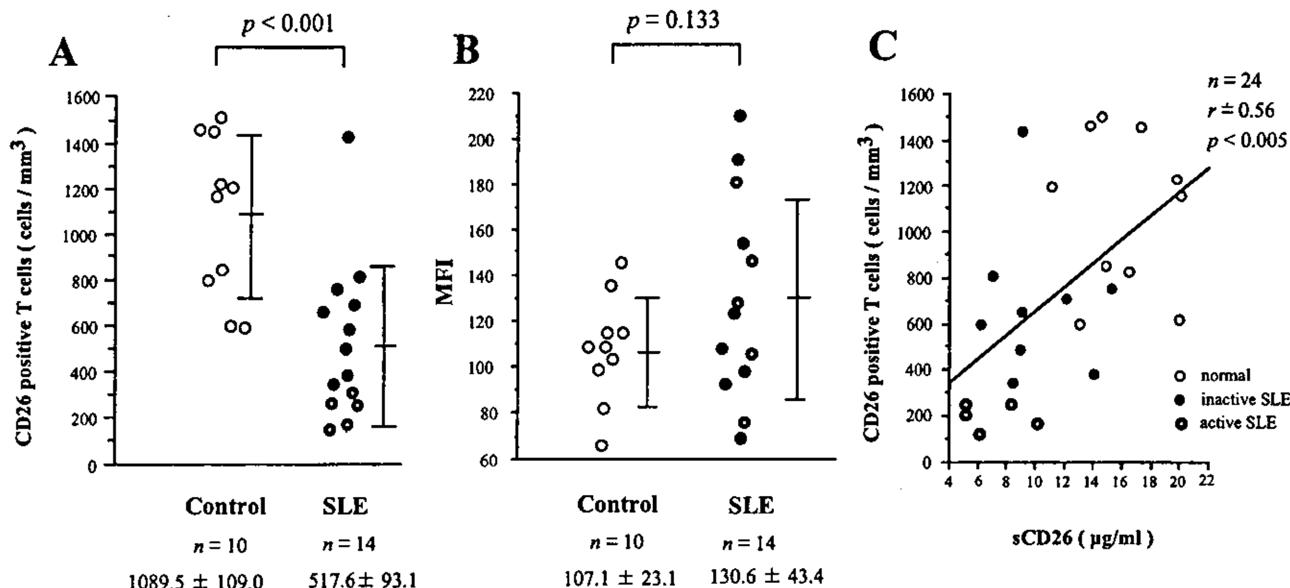

Figure 4. CD26 surface expression on peripheral blood T cells from patients with SLE. A. Levels of CD26 positive T cells were significantly decreased compared to those in healthy controls ($p < 0.001$). B. There was no statistically significant difference in mean fluorescence intensity (MFI) values of CD26 on T cells between the 2 groups ($p = 0.133$). C. There was a significant correlation between serum sCD26 levels and levels of CD26 positive T cells from peripheral blood of SLE patients ($r = 0.56$, $p < 0.005$).

activity and total DPPIV-like activity in sera revealed that sCD26 associated DPPIV enzyme activity accounted for 55 ± 1.5% (n = 5) of total DPPIV-like enzyme activity in sera from not only healthy controls but also patients with SLE. This result suggested the sCD26 and its associated DPPIV activity was the primary factor in the serum DPPIV-like enzyme activity. Thus we were able to clarify the inverse correlation with the disease activity of SLE due to the evaluation of sCD26 and/or sCD26 associated DPPIV enzyme activity.

Immunoprecipitation and immunoblotting analysis confirmed the presence of CD26-specific immunoreactivity in sera. Iwaki-Egami, *et al* purified CD26 from human serum and showed that serum CD26 has a slightly smaller molecular weight than the value predicted from its cDNA since it lacks the N-terminal 28 amino acid region encompassing the transmembrane domain[51]. Our data also suggest that the molecular weight of serum sCD26 is almost identical to that of the CHO expressed recombinant CD26, which has the 28 amino acid deletion at the N-terminal end (Figure 2). Since our analysis was done in reducing and denaturing conditions, we cannot rule out the possibility of an apparent loss of immunogenicity for this molecule due, for example, to protein modification and proteolysis. However, the fact that DPPIV enzyme activity significantly correlated with serum levels of sCD26 supports the notion that an observed decrease in serum sCD26 levels from patients with SLE was due to an actual reduction in the amount of protein. Further studies are planned to elucidate these issues.

Regarding possible clinical factors that might influence serum levels of sCD26, we analyzed the correlation of sCD26 levels with clinical data, e.g., age, sex, disease duration, daily dosage of prednisolone as a factor for medication, the erythrocyte sedimentation rate or C-reactive protein measure for inflammation, immunoglobulins, complement levels or lymphocyte count as an indicator for immunological function, renal function, liver function, and others. As shown in Table 2, we found no significant correlation with the various clinical factors except for anti-U1-RNP antibody. Our analysis of a potential relationship between serum sCD26 levels and various clinical features of SLE revealed no significant correlation between serum sCD26 levels and organ involvement of SLE, e.g., renal disease, hematologic disease, etc. Similarly, there was no significant correlation between serum sCD26 levels and Raynaud's phenomenon, arthritis, pulmonary involvement, or muscle involvement. Thus we could not determine why anti-U1-RNP antibody levels had significant correlation with serum levels of sCD26. As well, serum levels of sCD26 were negatively correlated with the SLEDAI, which is one of the representative markers of overall disease activity of SLE (Figure 3B). Serial determination of serum sCD26 levels revealed that changes in serum sCD26 levels reflected changes in SLE disease activity (Figure 3C). It is thus conceivable that a decrease in serum sCD26 levels is an important indicator of an ongoing SLE related pathological process, regardless of particular organ involvement.

Recently, Cordero, *et al* reported that sCD26 in patients with rheumatoid arthritis (RA) was significantly reduced

and related to disease activity[52]. We have also measured sCD26 levels/DPPIV in sera from patients with RA as disease controls. They were also reduced compared with healthy controls (sCD26 level 10.5 ± 3.0 µg/ml, DPPIV enzyme activity 6.0 ± 1.7 µmol/min, n = 40). Reduction of sCD26/DPPIV has been reported in patients with not only collagen disease but also several kinds of cancer[53-55], major depression[56,57], and human immunodeficiency virus (HIV) infection[50]. As one possible mechanism, impaired immune response was proposed to explain the reduction of sCD26/DPPIV in such diseases. Similarly, since sCD26/DPPIV levels appear to reflect disease activity of SLE, sCD26/DPPIV may have some association with the altered immune status in SLE, although reduction of serum sCD26/DPPIV is not specific for SLE.

Serum DPPIV activity was also evaluated in mouse models of lupus, in addition to human SLE[58,59]. One group reported DPPIV enzyme activity was decreased in the serum of NZB mice[58]. In contrast, serum DPPIV activity in MRL/lpr mice increased markedly in parallel with accelerated levels of DPPIV+ lymphocytes, reflected by increasing lymphadenopathy[59]. While both strains of mice represent animal models of human SLE, the reason for the relative difference in DPPIV enzyme activity between these 2 strains is unclear. One possible explanation may be that MRL/lpr mice have increased numbers of DPPIV+ lymphocytes, whereas NZB mice have a decreased number of lymphocytes[58].

Although sCD26 is present in the serum of healthy controls as well as patients with SLE, RA[52], and HIV-I infection[50], its origin has not been identified. Theoretically, soluble CD26 can be produced either by shedding of the membrane form or through an alternative mRNA splicing mechanism. Our previous studies indicate that CD26 does not undergo alternative splicing[3]. We also found that the level of CD26 positive T lymphocytes but not total or CD3 positive T lymphocytes was significantly correlated with serum sCD26 levels and SLEDAI score (Figure 4C). While CD26 is expressed on a variety of tissues in addition to lymphocytes, including kidney, intestine, and bile duct, the finding that sCD26 levels did not correlate with organ involvement of SLE would suggest that serum sCD26 is derived from CD26 positive T cells.

What is the mechanism behind the observed depletion of CD26 positive T cells? One possibility may involve internalization of CD26 molecules on T cells of patients with active SLE. We have observed[19] that CD26 expression is modified by M6P in activated T cells with resultant internalization of CD26 via M6P receptor. Although the overall level of CD26 expression on T cells is significantly decreased in patients with active SLE, a certain population of CD26 positive T cells might be activated, resulting in our observation of high mean fluorescence intensity values for T cell CD26 expression (Figure 4). Another scenario may involve recruitment of CD26 positive T cells to particular inflammatory sites in patients with active SLE, as it has been reported that CD26 positive T cells are predominantly found in affected joints in patients with RA[60]. Moreover, adenosine deaminase (ADA) is one of the association molecules of CD26[17,18]. Dysfunction of ADA may trigger lymphopenia in SLE due to the accumulation of adenosine. Although the precise mechanism remains unclear, the depletion of CD26 positive T cells may result in a corresponding decrease in serum sCD26 levels.

Studies have shown that many lymphocyte cell surface molecules are released from cell surfaces, with their levels being increased in the sera of patients with active SLE. Our findings that patients with active SLE exhibit low levels of sCD26 molecules are therefore in contrast with the high serum levels of other soluble cell surface molecules detected in such patients.

Depletion of sCD26 might also occur as a result of increased uptake and/or turnover by the reticular endothelial system in active SLE. Of interest is that we observed a case complicated with hemophagocytic syndrome. *In vitro* experiments by our group showed that cellular uptake of soluble CD26 was mediated mostly by macrophages, as compared to T and B lymphocytes. Following uptake by macrophages, sCD26 molecules were subsequently transported into lysozomes via M6P/IGFIIR. Importantly, macrophage surface expression of CD86 but not CD80 was upregulated during this process[21]. Since CD80/86 and CD28 interaction plays a potent role in immune system costimulation, these results strongly suggested that macrophage uptake of sCD26 may contribute to immune activation by antigen-presenting cells, leading eventually to stimulation of autoantibody-producing B cells in patients with SLE. In this scenario, sCD26 uptake by macrophages would contribute to the observed low serum sCD26 levels in SLE.

Serum levels of sCD26 may be involved in the pathophysiology of SLE and appear to be useful as a new disease activity measure of SLE. Studies to define the underlying mechanisms of the decrease in serum sCD26 levels may contribute to our understanding of immunological abnormalities in SLE, and its pathogenesis.

ACKNOWLEDGMENT

The authors would like to express appreciation to A. Kuribara for her technical assistance.

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Eur. J. Immunol. 2002. 32: 2188–2198

Lipid rafts as the signaling scaffold for NK cell activation: tyrosine phosphorylation and association of LAT with phosphatidylinositol 3-kinase and phospholipase C-γ following CD2 stimulation

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Natural killer (NK) cells participate in both innate and adaptive immunity through the prompt secretion of cytokines and ability to lyse virally infected cells or tumor cells. Although it has been well understood that lipid rafts (rafts) and a raft-associated linker for activation of T cells (LAT) plays a central role in TCR signal transduction, there are still great gaps in our knowledge of the molecular events involved in NK cell activation. We show here that CD2 and rafts became polarized to the site of NK cell activation by CD2 cross-linking or target cell binding using confocal microscopy, and LAT and a significant amount of CD2 colocalized in raft fractions of sucrose-density gradient from an NK cell line, NK3.3. CD2 cross-linking strongly induced tyrosine phosphorylation of LAT, resulting in increased association with phosphatidylinositol 3-kinase (PI 3-K) and phospholipase C-γ1 (PLC-γ1). *In vitro* binding studies using glutathione S-transferase fusion proteins demonstrated that a large portion of the association between LAT and PI 3-K or PLC-γ1 was mediated through their SH2 domains in tyrosine phosphorylation-dependent manner. Furthermore, disruption of lipid rafts by cholesterol depletion from cell membranes using methyl-β-cyclodextrin markedly reduced LAT tyrosine phosphorylation and NK cell functions, including cytotoxicity and granule exocytosis. These results document that modulation of raft integrity by aggregation of NK cell activating receptors, which leads to the formation of complexes of LAT with PI 3-K and PLC-γ1, is essential for the NK cell lytic mechanisms.

Received	5/3/02
Revised	10/5/02
Accepted	4/6/02

Key words: NK cell / Lipid raft / CD2 / Linker for activation of T cells / Cytotoxicity

1 Introduction

One mechanism used by NK cells or CTL for the destruction of target cells involves the granule exocytosis pathway, which utilizes the lytic mediators perforin and several proteases (granzymes) contained within cytoplasmic granules [1]. The interaction between ligands on the target cells and activating receptors on NK cells including CD2, CD16, and chemokine receptors, initiates a series of biochemical events responsible for the redistribution of the granules and concentration of their constituents at the area of cell to cell contact, where the lytic mediator are released, resulting in target cells lysis [2–5]. By contrast, NK cell activation is blocked when killer immunoglobulin-like receptors (KIR) on the surfaces of NK cells engage MHC class I molecules on resistant target cells [6–8]. It is becoming increasingly clear that the final outcome of NK cell activity results from a balance

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Abbreviations: LAT: Linker for activation of T cells **PLC:** Phospholipase C **PI 3-K:** Phosphatidylinositol 3-kinase **p85:** p85 subunit of PI 3-K **SH:** Src homology **pTyr:** Phosphotyrosine **GST:** Glutathione S-transferase **ITIM:** Immunoreceptor tyrosine-based inhibition motifs **MβCD:** Methyl-β-cyclodextrin **BLTE:** N^{α}-Benzyloxycarbonyl-L-lysine thiobenzyl ester

0014-2980/02/0808-2188$17.50+.50/0
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between the expression of activatory and inhibitory receptors and their ligands [9, 10].

Recent evidence suggests that cholesterol-sphingolipid microdomains, designated as lipid rafts (rafts), detergent-resistant membranes, detergent-insoluble glycosphingolipid-enriched membranes or glycosphingolipid-enriched membrane, play an important role in T cell signal transduction, because signal-transducing molecules including the *src*-family kinases *Lck* and *Fyn*, CD4, and LAT (linker for activation of T cells) show high and specific activity in the detergent-resistant membrane compartment and rafts [11, 12]. LAT is a predominantly cytoplasmic membrane protein with two cysteine residues just below the transmembrane region and is targeted to the rafts by palmitoylation of cysteines [13, 14]. LAT also contains multiple tyrosine-based motifs which, when phosphorylated by *ZAP-70* or *Syk*, initiate the assembly with SH2 domain-containing signaling proteins such as phosphatidylinositol 3-kinase (PI 3-K), phospholipase C-γ1 (PLC-γ1) and Grb2, and allows subsequent extensions of the signaling scaffold [15–17]. Thus, it is well documented that rafts and LAT play a central role in TCR signal transduction. However, the molecular events of raft and LAT involved in NK cell activation, leading to tumor cell cytotoxicity, remain to be elucidated.

CD2 is believed not only to function in adhesion through binding its ligands CD48, CD58, and CD59, but also to generate transmembrane signals during the activation of T cells and NK cells, including tyrosine phosphorylation, Ca^{2+} flux, intracellular cyclic AMP generation, and the activation of various signaling molecules [18, 19]. We have previously reported that CD2 cross-linking activates NK cells and increases tyrosine phosphorylation of cellular proteins through *Syk* activation, leading to increased association among signaling molecules, *i.e.* PI 3-K/Shc or Grb2/Cbl [3, 20]. We have also observed that CD2 stimulation, but not IL-2 stimulation, induces tyrosine phosphorylation of LAT in an NK cell line, NK3.3 [21].

Using the NK cell line, NK3.3 to assess the relationship between raft integrity and NK cell activation signaling, we first analyzed the geometry of rafts and CD2 by laser scanning confocal microscopy and sucrose-gradient centrifugation. We then examined the effects of CD2 cross-linking on tyrosine phosphorylation and association of LAT with PLC-γ1 and PI 3-K, and the effects of lipid raft disruption by cholesterol depletion from cell membranes.

2 Results

2.1 Raft polarization at the activation sites of NK3.3 cells by CD2 cross-linking or target cell binding

Although accumulating evidence supports an important role for rafts in T cell-mediated signaling, less is known about raft function in NK cell activation. Lou et al. [6] have reported that rafts become polarized to the sites of binding between NK cells and NK-sensitive target cells, and that the killer cell inhibitory receptor-associated phosphatases, SHP-1, blocks redistribution of rafts in NK resistant target cells. Fassett et al. [8] have reported that NK lipid raft polarization and inhibitory NK immune synapse formation occur by fundamentally distinct mechanisms. To investigate the linking between raft polarization and NK cell activation, we used confocal microscopy to examine whether CD2 cross-linking induces raft polarization in NK3.3 cells after incubation with FITC-conjugated cholera toxin B subunit (CTx), which binds GM1 ganglioside, a marker of glycolipid-enriched rafts. Cells were then treated with PE-conjugated anti-CD2 murine mAb on ice for 30 min, washed, and stimulated with polybeads pretreated with rabbit anti-mouse IgG Ab or normal rabbit serum (NRS) at 37°C for 30 min. As shown in Fig. 1, rafts (green) and CD2 (red) are dispersed throughout the plasma membrane in resting NK3.3 cells (data not shown) and in NK3.3 cells stimulated with beads pretreated with NRS. In contrast, when cells were stimulated by CD2 cross-linking, both rafts and CD2 redistributed to form similarly shaped clusters of identical cellular localization and polarity that colocalize (yellow).

To examine the localization of rafts and CD2 after effector-target cell conjugation NK3.3 cells were labeled with FITC-CTx and PE-CD2 mAb, and mixed with K562 target cells. Fig. 2A depicts representative photographs of NK3.3 and K562 conjugates, showing reorganization of lipid rafts and CD2 at the contact area. We further examined whether CD58 (a ligand for CD2) on K562 cells polarized at the area of contact with NK3.3 cells. NK3.3 cells labeled with FITC-CTx and K562 cells labeled with PE-CD58 were mixed and examined by confocal microscopy. Fig. 2B clearly shows the reorganization of CD58 on K562 cells as well as lipid rafts on NK3.3 cells at the contact area.

2.2 Colocalization of CD2 and LAT in raft fractions of sucrose gradient of NK3.3 cells

It has been reported that rafts serve as platforms for glycosylphosphatidylinositol (GPI)-anchored proteins (*e.g.* CD14, CD16b, CD24, CD28, CD48, CD58, CD59, CD67,

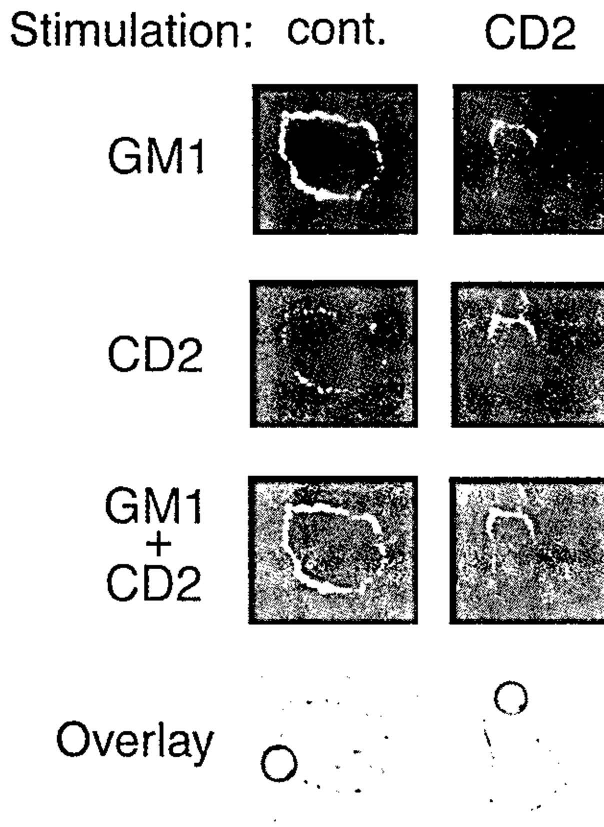

Fig. 1. Copolarization of rafts and CD2 following CD2 cross-linking. NK3.3 cells were first stained with FITC-conjugated cholera toxin B subunit (GM1), then incubated with PE-conjugated CD2 mAb. After stimulation with polybeads pretreated with normal rabbit serum (cont.) and rabbit anti-mouse IgG Ab (CD2), cells were analyzed by confocal microscopy. Photographs of cells shown here represent the majority of cells displaying cell surface staining patterns observed in these experiments. GM1 (rafts, green), CD2 (red), copolarization of rafts and CD2 (yellow), overlay (transmission light image and fluorescent image).

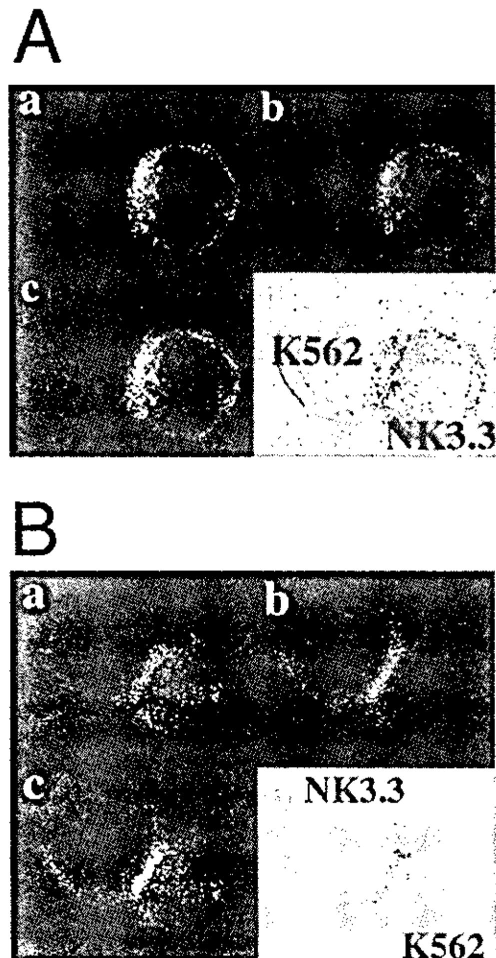

Fig. 2. Copolarization of rafts and CD2 at the contact site of NK and target cell. (A) Copolarization of rafts and CD2 on NK cells. NK3.3 cells labeled with FITC-CTx and PE-CD2 mAb were mixed with K562 cells. (B) Redistribution of CD2 on NK cells and CD58 on target cells to the contact site. NK3.3 cells labeled with FITC-CTx and K562 cells labeled with PE-CD58 were mixed. Mixtures were incubated at 37°C for 5 min, and conjugates were analyzed by confocal microscopy. Photographs shown here represent the majority of binding cells displaying cell surface staining patterns observed in these experiments.

CD73, CD87 and CD157), some transmembrane proteins (*e.g.* LAT, CD4, CD8, CD26, CD36 and CD44), surface receptors (*e.g.* FcεRI, FcαR and BCR) and doubly acylated protein tyrosine kinases of the *Src* family (*Lyn, Lck, Fyn* and *Hck*) [11, 22]. Recently, CD2 has been also reported to localize in raft fractions in mouse and human T cells [23 and 24, respectively]. We have previously reported that CD2 transduces NK cell activation signals, including tyrosine phosphorylation and complex formation of various adapter proteins, such as Grb2, Crk, Cbl, Shc and LAT [3, 20, 21]. Therefore, we examined whether CD2 and LAT co-exist in raft fractions of sucrose gradients from NK3.3 cells.

Raft fractions were isolated from Triton X-100 lysates of NK3.3 cells after equilibrium density gradient centrifugation in a sucrose density gradient, localized at the interface between the top (5%) and middle (30%) sucrose layers [25], corresponding to fractions 4 to 6 in our density gradient fraction [26]. The Triton X-100-soluble materials remained at the bottom of the gradient in our fractions 11 and 12. We confirmed the enrichment of glycosphingolipids in fractions 4 to 6 by reactivity with cholera toxin (Fig. 3, upper), and that almost all the LAT was recovered from the same raft fractions of NK3.3 cells (Fig. 3, middle), consistent with previous reports in T cells [11, 14]. Furthermore, we found that a significant

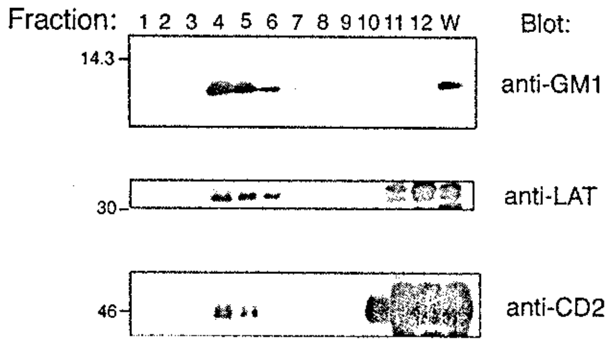

Fig. 3. Co-localization of CD2 and LAT in raft fractions. The lysate from NK3.3 cells was fractionated on a sucrose density gradient. Equal aliquots from each fraction were resolved on SDS-PAGE, and proteins were analyzed on immunoblots stained with cholera toxin B subunit reactive with GM1, anti-LAT Ab and anti-CD2 mAb as indicated.

amount of CD2 was in the raft fraction before CD2 cross-linking, although a major part of CD2 was in the Triton X-100-soluble fractions (Fig. 3, bottom).

2.3 CD2-mediated tyrosine phosphorylation of LAT in NK3.3 cells

It has been reported that LAT is targeted to rafts by palmitoylation of the cysteines just below the transmembrane region and that LAT is implicated in linking tyrosine-phosphorylated molecules to downstream signals under various conditions of stimulation [13, 14]. We and others have reported that cross-linking of CD2 or CD16 activates *Syk* and enhances tyrosine phosphorylation of cellular proteins in NK cells [3, 27, 28]. Since LAT is a substrate for *ZAP-70* and *Syk,* we investigated whether LAT is tyrosine-phosphorylated in NK3.3 cells following CD2 stimulation. NK3.3 cells were stimulated with various Ab, solubilized by lysing buffer with Brij 96, which has been reported to disrupt lipid rafts [13]. LAT were immunoprecipitated and subjected to SDS-PAGE and anti-phosphotyrosine (anti-pTyr) immunoblotting.

Cross-linking of CD2 or CD16 induced marked tyrosine phosphorylation of LAT, while treatment with control IgG1, anti-LFA-1α, β, or CD28 did not enhance tyrosine phosphorylation of LAT (Fig. 4A). Tyrosine phosphorylation of LAT induced by CD2 cross-linking was Ab

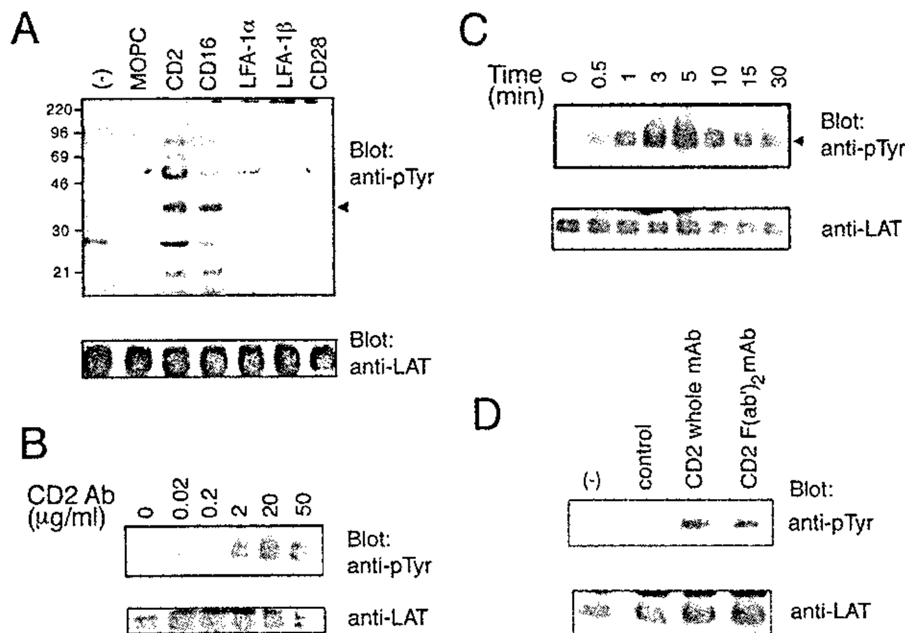

Fig. 4. Tyrosine phosphorylation of LAT following CD2 cross-linking. NK3.3 cells (10^7) were treated with the indicated Ab and stimulated using polybeads coupled to rabbit anti-mouse IgG Ab at 37°C. Detergent-soluble proteins were immunoprecipitated with anti-LAT Ab. The membrane was immunoblotted with anti-pTyr mAb (anti-PTy), and then stripped and immunoblotted with LAT Ab. (A) Effects of cross-linking using various Ab (each 2 μg/ml) on LAT tyrosine phosphorylation. (B) Concentration-dependent effects of CD2 mAb on LAT tyrosine phosphorylation. (C) Time-course study of LAT tyrosine phosphorylation by CD2 cross-linking. (D) Effects of $F(ab')_2$ fragment of CD2 mAb on LAT tyrosine phosphorylation.

concentration-dependent (Fig. 4B), and results of a time course study revealed that tyrosine phosphorylation of LAT occurred within 30 s with maximum response achieved at 3 min (Fig. 4C). To exclude the possibility that nonspecific binding of the Fc portion of CD2 mAb to the FcγRIII (CD16) may cause LAT tyrosine phosphorylation, we examined the effect of $F(ab')_2$ fragment of CD2 mAb on tyrosine phosphorylation of LAT. Fig. 4D shows that cross-linking using the $F(ab')_2$ fragment of CD2 mAb also induced tyrosine phosphorylation of LAT to the same extent as whole CD2 mAb. The membranes were stripped and reprobed with anti-LAT Ab, and the results revealed that comparable amounts of LAT were immunoprecipitated from lysates obtained from NK3.3 cells subjected to each treatment (Fig. 4A–D). We concluded that multivalent cross-linking of CD2 itself was sufficient to transduce activation signals in NK3.3 cells.

2.4 CD2-mediated association of LAT with PI 3-K and PLC-γ in NK3.3 cells

PI 3-K is known to be involved in various cellular functions [29], including the granule exocytosis pathway in NK cells [3, 30, 31]. Therefore, we investigated whether LAT associates with PI 3-K in NK3.3 cells following CD2 stimulation. CD2 cross-linking enhanced tyrosine phosphorylation of LAT and the p85 subunit of PI 3-K. Results of blotting of the membrane with anti-p85 and anti-LAT Ab revealed that CD2 stimulation induced association between PI 3-K and LAT (Fig. 5A). *In vitro* binding assays using N-terminal or C-terminal SH2-GST fusion proteins of p85 show that LAT associated with both SH2-GST fusion proteins is increased and tyrosine-phosphorylated following CD2 cross-linking (Fig. 5B).

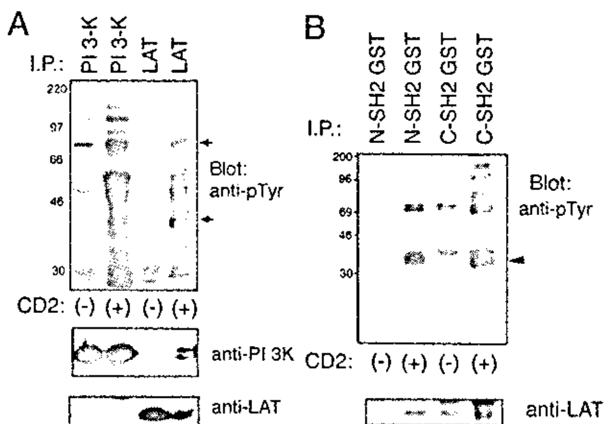

Fig. 5. Interaction between LAT and PI 3-K following CD2 stimulation. (A) Association of LAT with PI 3-K. NK3.3 cells were stimulated by CD2 cross-linking and lysates were immunoprecipitated with anti-LAT and anti-PI 3-K Ab. Tyrosine phosphorylation and association of LAT and PI 3-K were detected by immunoblot. (B) Association of LAT with PI 3-K analyzed by an *in vitro* binding assay. After stimulation, lysates were incubated with N-terminal and C-terminal SH2-GST fusion proteins of PI 3-K, p85, for 2 h at 4°C. Interacting complex were immunoprecipitated with anti-GST mAb.

It has also been reported that PLC-γ1 is involved the granule exocytosis pathway in NK cells and that tyrosine-phosphorylated LAT binds to PLC-γ1 [13, 32]. CD2 cross-linking enhanced tyrosine phosphorylation of LAT associated with PLC-γl and blotting of the membrane with anti-LAT Ab revealed that CD2 stimulation increased the association between PLC-γ1 and LAT (Fig. 6A). Moreover, results of *in vitro* binding assays using N-terminal and C-terminal SH2-GST or SH3-GST fusion proteins of PLC-γ1 show that LAT associated with both SH2-GST fusion proteins are increased and tyrosine was phosphorylated following CD2 cross-linking, despite that LAT associated with SH3-GST fusion proteins are neither increased nor tyrosine-phosphorylated (Fig. 6B).

2.5 Effect of cholesterol depletion on NK cell activity

The model for raft structure postulates an important role for cholesterol as a spacer to link sphingolipids together in the exoplasmic leaflet of the bilayer [33]. Disruption of rafts using the cholesterol-chelating reagent, methyl-β-cyclodextrin (MβCD), has been used to study the role of rafts in TCR signaling [34, 35]. To investigate the importance of raft polarization in NK cell activation, NK3.3 cells were pretreated with the indicated concentration of MβCD for 20 min at 37°C before the assay. Cells treated in this manner remained viable, as determined by trypan blue staining (data not shown). NK3.3 cells were stimulated by CD2 cross-linking, lysed, and immunoprecipitated with anti-LAT Ab. As shown in Fig. 7, CD2-induced tyrosine phosphorylation of LAT was inhibited by treatment of cells with MβCD in dose-dependent manner.

Finally, we examined the effects of MβCD on NK cell cytolytic activity and granule release. After treatment with MβCD, NK3.3 cells were incubated with calcein AM-labeled K562 target cells for 4 h. Fluorescence of supernatants was assayed and the results were expressed as lytic $units_{20\%}$. Fig. 8A shows that MβCD dramatically inhibited NK cytolytic activity in a dose-dependent manner. One key mechanism by which NK cells lyse target cells is through the granule exocytosis pathway, which primarily uses the lytic mediators, perforin and granzy-